COMPARISON OF THE BINDING ABILITY OF HEMOCYANIN AND SERUM ALBUMIN FOR ORGANIC IONS

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Introduction

The presence of hemocyanin in a dispersed form in the plasma of invertebrates suggests that this protein may act not only as a respiratory pigment but also as a transport vehicle for small ions. In mammalian plasma these functions are divided between two proteins, hemoglobin and serum albumin, respectively. The importance of the "vehicle function" of plasma albumin in facilitating or hindering the distribution of substances amongst various tissues and organs has been pointed out by Bennhold (1938) and by Davis (1946). No investigations have been reported on the interactions of hemocyanins with small ions, other than those involved in the respiratory function (Redfield, 1934; Dawson and Mallette, 1945).

A series of studies has been made, therefore, on the binding of several organic dye ions by the serum of the horseshoe crab, *Limulus polyphemus*. Parallel investigations for some anionic substances and bovine serum albumin have been reported previously (Klotz, 1946; Klotz and Walker, 1947). In the absence of quantitative data on the binding of cationic dyes by albumin, some experiments were carried out also with these substances and bovine serum albumin.

Methods and Materials

Quantitative measurements of the extent of binding by the proteins were made by the dialysis-equilibrium technique (Klotz, Walker and Pivan, 1946). A cellophane bag containing a measured quantity (10 cc.) of the serum, or of the solution of pure protein, was immersed in a large test tube containing a definite volume (20 cc.) of the solution of the small ion at a known concentration. The bag, impermeable to protein, was mounted on a glass frame from which a small bead was suspended by a Fiberglas thread into the protein solution. The test-tube was placed in a shaking device for twenty-four hours for the attainment of equilibrium. The bead in the protein solution served as a stirring device for the contents of the bag and thereby hastened materially the attainment of equilibrium. The bag was then removed and the external solution analyzed colorimetrically for the ion.

For each ion concentration, a control tube was prepared also, which differed from the primary tube only in that the former contained buffer rather than a protein solution or serum inside the bag. By this method it was possible to minimize any errors arising from binding of the ion by the cellophane membrane.

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For each ion investigated, a preliminary series of experiments was run to ascertain the time necessary for the attainment of equilibrium. With the bead device described, a period of twenty-four hours was found to be more than sufficient in every case. A typical set of results for the cationic dye, chrysoidine, is illustrated by the data in Table I. The other substances showed similar behavior.

TABLE I

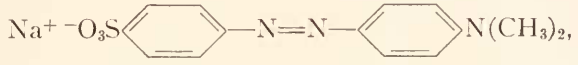
Time for attainment of equilibrium in binding of chrysoidine

Time	Conc. of dye outside bag
0.00 hours	9.2×10^{-5} moles/liter
6.25	5.2
18.75	5.2
23.5	5.2

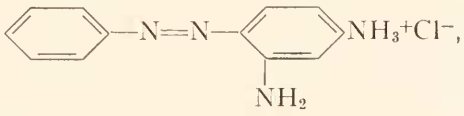
Limulus serum was obtained from a heart puncture of the animal.[3] Coagulation was permitted to occur at room temperature for about fifteen minutes. The serum obtained by subsequent filtration was diluted to four times its initial volume and stored in a refrigerator until used.

Bovine serum albumin was recrystallized material obtained from Armour and Company. Solutions of 0.2 and 2 per cent concentration were used in the binding experiments.

The methyl orange,



was a commercial sample of reagent grade. The chrysoidine,



was a commercial sample.

The dialysis experiments at pH 5.0 were carried out in 0.1 M acetate buffer solutions and those at pH 5.2 in 0.2 M phosphate. The pH values were measured with a Coleman glass electrode. Colorimetric analyses were carried out with a Klett-Summerson photoelectric colorimeter. All equilibria were attained in a water bath at $25 \pm 2°$ C.

RESULTS AND DISCUSSION

Data on the binding of methyl orange, a typical aromatic organic anion in its protein-binding properties (Klotz, 1946), by *Limulus* serum at a pH of 5.0 are summarized in Table II. It is evident that appreciable binding occurs with *Limulus* serum also, and that the extent of combination increases with the concentration of free dye. Relatively wide variations in the data are observed with this serum, however, probably because of differences between samples.

If one assumes that the serum contains about 4 per cent hemocyanin, then 10 cc. of the diluted solution within the bag contains approximately 0.1 gram of protein,

[3] We are indebted to Miss Marguerite Webb for technical advice.

TABLE II

Binding of methyl orange by Limulus *serum, pH 5.0, acetate buffer*

Conc. of free dye	Moles dye bound by hemocyanin in 10 cc. of diluted serum
1.5×10^{-5} M	0.5×10^{-7}
2.7	1.6
3.8	2.1
3.8	2.1
5.3	3.3
5.6	2.7
5.8	1.8
10.0	6.9
10.8	3.9
10.8	6.3

and at a concentration of 1×10^{-4} molar free dye, approximately 0.5×10^{-5} mole of methyl orange would be bound by one gram of hemocyanin. This is about one-tenth the degree of binding observed with bovine albumin (Klotz, Walker and Pivan, 1946) where approximately 6×10^{-5} mole of methyl orange is bound by one gram of protein at a free dye concentration of 1×10^{-4} molar.

Several experiments were carried out with methyl orange and hemocyanin in a phosphate buffer at pH 5.2. The results obtained (Table III) indicate a slightly decreased degree of binding. However, the difference in the two pH's is too small to be attributed to changes in hydrogen-ion concentration for there may be small deviations due to differences in the ionic nature and strength of the buffer solutions.

TABLE III

Binding of methyl orange by Limulus *serum, pH 5.2, phosphate buffer*

Conc. of free dye	Moles bound dye
4.8×10^{-5} M	1.2×10^{-7}
6.1	1.6
8.6	2.4
11.9	3.6
17.6	9.3

In the case of albumin, there is strong evidence (Klotz and Walker, 1947) that electrostatic attraction between the organic anion and a positively-charged, quaternary nitrogen of one of the basic amino acids contributes a major portion of the binding energy. While the demonstration of a similar force in the hemocyanin complex requires further work on the effect of pH, it is, nevertheless, of interest to note that this protein like albumin is rich in the basic amino acids, histidine, lysine and arginine (Dawson and Mallette, 1945).

As an example of a cationic species, the dye chrysoidine was used at a pH of 5. No significant binding was observed, as is evident from the data in Table IV. The concentration of free cation was not reduced significantly by the presence of diluted *Limulus* serum inside the cellophane bag.

In the absence of comparable data for albumin, a series of binding experiments was carried out with chrysoidine and bovine serum albumin. The results obtained have been assembled in Table V. It is evident that chrysoidine is bound by albumin,

though only about one-tenth as strongly as is methyl orange. If the same decrease in binding in going from the anion, methyl orange, to the cation, chrysoidine, holds in hemocyanin as is found with albumin, it would require more precise techniques than were available to detect the presence of a chrysoidine complex of hemocyanin.

TABLE IV

Absence of binding of chrysoidine by Limulus *serum, pH 5.0*

Conc. of free dye in equilibrium with protein	Conc. of free dye in blank
5.8×10^{-5} M	6.1×10^{-5} M
17.8	16.3
27.0	28.8
32.8	33.0
39.0	39.5
44.5	44.0

TABLE V

Binding of chrysoidine by bovine serum albumin, pH 4.7

Conc. of free dye	$\frac{\text{Moles bound dye}}{\text{Moles total albumin}}$
1.1×10^{-5} M	0.0523
2.4	0.136
3.8	0.188
5.8	0.279
6.0	0.294
12.0	0.577
12.7	0.652
19.8	0.871
19.8	1.09
24.5	1.05
29.5	1.31
34.5	1.42

The binding data for chrysoidine with bovine albumin can also be expressed analytically by the equation

$$\frac{\text{moles protein}}{\text{moles bound dye}} = \frac{1}{k\,n}\,\frac{1}{(\text{free dye})} + \frac{1}{n}$$

where k represents the intrinsic binding constant and n, the maximum number of chrysoidine ions which can be bound by a single albumin molecule. As has been shown previously (Klotz and Walker, 1947) this equation is derivable from the law of mass action for the ideal situation in multiple binding, i.e. one in which a bound ion exerts no electrostatic influence on successively bound ions. Thus within the precision of the present experiments, chrysoidine fulfills this ideal condition. The maximum number of sites, n, available to chrysoidine turns out to be 16. The intrinsic binding constant equals 3.1×10^2. From these values it follows (Klotz, Walker and Pivan, 1946) that the energy of binding of the first chrysoidine ion is about 5,000 calories/mole in comparison to 5,960 calories/mole for methyl orange.

Attempts were made also to obtain some information on the binding of methylene blue by albumin and hemocyanin, respectively. Unfortunately, the persistence

of micelle formation by this dye even at exceedingly low concentrations made it difficult to interpret the data. Difficulties were also encountered in preliminary experiments on the binding of ferric iron, apparently because of oxidation of the cellophane membranes in the presence of this ion at the relatively high acidities (pH 3) which were necessary to avoid formation of colloidal iron oxide.

Conclusions

The results obtained indicate that hemocyanin is capable of forming complexes with organic anions though the binding affinity is less than that observed with bovine albumin. Thus in addition to its function as a respiratory pigment, hemocyanin may be capable of acting as a vehicle in the transport of anions in the blood of invertebrates, as an agent for conserving desirable substances as the blood passes through the excretory organs and as a buffer against the effects of cytotoxic agents (Davis, 1946). In all of these actions, however, the present experiments indicate that its effectiveness is much less than that of serum albumin.

Summary

1. Hemocyanin forms complexes with organic anions, such as methyl orange, though with an affinity of about one-tenth that observed with the same dye and serum albumin.

2. No binding was observed between hemocyanin and the cationic dye, chrysoidine. Under comparable conditions bovine serum albumin combined with chrysoidine, thought quantitative calculations indicate an energy of binding about 1,000 calories less than that observed for albumin-methyl orange complexes.

3. The data indicate that hemocyanin acts not only as a respiratory pigment in the blood of invertebrates but also may serve to a limited extent in the distribution and conservation of organic ions among various organs and tissues.

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